MOLECULAR AND CELLULAR BIOLOGY, July 1986, p. 2490–2499
0270-7306/86/072490-10$02.00/0

Vol. 6, No. 7

PEP4 Gene of *Saccharomyces cerevisiae* Encodes Proteinase A, a Vacuolar Enzyme Required for Processing of Vacuolar Precursors

GUSTAV AMMERER,[1]† CRAIG P. HUNTER,[2]‡ JOEL H. ROTHMAN,[2] GENA C. SAARI,[1] LUIS A. VALLS,[2] AND TOM H. STEVENS[2]*

Zymogenetics, Inc., Seattle, Washington 98103,[1] and Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403[2]

Received 26 December 1985/Accepted 7 March 1986

The proteinase A structural gene of *Saccharomyces cerevisiae* was cloned by using an immunological screening procedure that allows detection of yeast cells which are aberrantly secreting vacuolar proteins (J. H. Rothman, C. P. Hunter, L. A. Valls, and T. H. Stevens, Proc. Natl. Acad. Sci. USA, 83:3248–3252, 1986). A second cloned gene was obtained on a multicopy plasmid by complementation of a *pep4-3* mutation. The nucleotide sequences of these two genes were determined independently and were found to be identical. The predicted amino acid sequence of the cloned gene suggests that proteinase A is synthesized as a 405-amino-acid precursor which is proteolytically converted to the 329-amino-acid mature enzyme. Proteinase A shows substantial homology to mammalian aspartyl proteases, such as pepsin, renin, and cathepsin D. The similarities are most striking between the precursors of proteinase A and human lysosomal cathepsin D; these similarities may reflect not only analogous functions but also similar processing and intracellular targeting mechanisms for the two proteins. The cloned proteinase A structural gene, even when it is carried on a single-copy plasmid, complements the deficiency in several vacuolar hydrolase activities that is observed in a *pep4* mutant. A strain carrying a deletion in the genomic copy of the gene fails to complement a *pep4* mutant of the opposite mating type. Genetic linkage data demonstrate that integrated copies of the cloned proteinase A structural gene map to the *PEP4* locus. Thus, the *PEP4* gene encodes a vacuolar aspartyl protease, proteinase A, that is required for the in vivo processing of a number of vacuolar zymogens.

The vacuole of *Saccharomyces cerevisiae* contains numerous hydrolytic enzymes (36). The biosynthesis of these enzymes follows a strategy (14, 31) that is similar to that used by mammalian cells in the biosynthesis of lysosomal enzymes (3). All vacuolar enzymes characterized thus far are glycoproteins that are synthesized as higher-molecular-weight zymogens (14). The best-characterized vacuolar enzyme, carboxypeptidase Y (CPY), is synthesized as a 532-aminoacid inactive precursor (9, 30a). This CPY precursor (proCPY) is transported from the site of synthesis at the endoplasmic reticulum (7, 31) through the Golgi apparatus to the vacuole (31), where the protein is found as the 421-amino-acid mature enzyme. Similarly, vacuolar proteinase A (PrA) and proteinase B (PrB) are also synthesized as precursors that are about 10 kilodaltons larger than the mature enzymes (21). Therefore, it is likely that most vacuolar enzymes are synthesized with a propeptide. This propeptide may serve both as a sorting determinant (30a) and to keep the enzyme inactive during transport (14).

The activities of many vacuolar hydrolyases are dependent on the allelic state of the *PEP4* locus (10). Cells carrying the pleiotropic *pep4-3* mutation lack CPY, PrA, PrB, vacuolar RNase, nonspecific alkaline phosphatase, and aminopeptidase I activities (10, 33). While there is disagreement over whether the precursor form of PrA (proPrA) accumulates in *pep4* cells (22, 27a, 41), *pep4* cells do accumulate precursor forms of CPY and PrB (10, 14). For CPY it has been shown that the precursor resides in the vacuole of such cells (31). These results have led to the hypothesis that the *PEP4* gene encodes a protease which is required for the processing of vacuolar protein precursors to their mature forms (14).

A nonpleiotropic class of mutations that affect only PrA activity has been described previously (23). These mutations define a single complementation group, *PRA1*. Because of their different phenotypes, it has been assumed that *PEP4* and *PRA1* represent different complementation groups, although the results of allelism tests have never been reported.

The PrA structural gene has recently been cloned and has been shown to complement the pleiotropic phenotype of the *pep4-3* mutation when it is carried on a multicopy plasmid (27a). A deletion created in the genomic copy of the PrA structural gene results in a pleiotropic phenotype; these cells lack CPY, PrA, PrB, and alkaline phosphatase activities (27a). Furthermore, the absence of CPY activity has been found to be due to an intracellular accumulation of unprocessed proCPY. We conducted further studies to ascertain the relationship between PrA and the *PEP4* gene product. In this paper we show that the *PEP4* gene encodes the vacuolar glycoprotein PrA. Thus, PrA either processes vacuolar precursors directly or is essential for the activation of a processing protease.

MATERIALS AND METHODS

Strains and materials. The yeast strains used in this work are described in Table 1. Strains were constructed by standard genetic manipulations.

* Corresponding author.
† Present address: Laboratory of Molecular Biology, Medical Research Council Center, Cambridge, CB2 2QH England.
‡ Present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309.

TABLE 1. Yeast strains used

Strain	Genotype	Source or reference
JHRY20-2C	*MATa his3-Δ200 ura3-52 leu2-3,112*	27a
JHRY20-2CΔ1	*MATa his3-Δ200 ura3-52 leu2-3,112 pep4-Δ1::URA3*	27a
JHRY20-3CΔ1	*MATa his3-Δ200 ura3-52 lys2-801 leu2-3,112 pep4-3 prc1-Δ3::HIS3*	This work
JHRY85	Diploid (JHRY20-2C × SF838-5A)	This work
JHRY86	Diploid (JHRY20-2CΔ1 × SF838-5A)	This work
JHRY88	Diploid (JHRY20-2C × SF838-1D)	This work
JHRY89	Diploid (JHRY20-2CΔ1 × SF838-1D)	This work
JHRY90	Diploid (SF838-9DR2L1 × SF838-1D)	This work
SF657-2D	*MATa his4 ura3-52 leu2-3,112 pep4-3*	R. Schekman
SF838-1D	*MATα ade6 his4-519 ura3-52 leu2-3,112 pep4-3*	R. Schekman
SF838-5A	*MATα ade6 ura3-52 leu2-3,112*	R. Schekman
SF838-9DR2L1	*MATa his4-519 ura3-52 lys2 leu2-3,112 pep4-3*	R. Schekman; this work
TSY6-7D	*MATa his4-519 ura3-52 leu2-3,112 pep4-3*	This work
ZA447[a]	*MATα ura3-52 leu2-3,112 bar1*	This work
ZA512[a]	*MATα ura-3-52 leu2-3,112 bar1 pep4-Δ2::LEU2*	This work
ZA515[a]	*MATα ura3-52 leu2-3,112 bar1 pep4-Δ1::URA3*	This work
ZA521[a]	*MATa ura3-52 leu2-3,112 bar1 pep4-Δ1::URA3*	This work

[a] Strains ZA447, ZA512, ZA515, and ZA521 are isogenic.

All reagents used in liquid enzymatic assays, the PrB plate assay, and the CPY plate assay were obtained from Sigma Chemical Co., St. Louis, Mo. The enzymes used in recombinant DNA manipulations were obtained from New England BioLabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Inc., Gaithersburg, Md. Carrier-free ^{35}S-labeled $H_2{}^{35}SO_4$ was obtained from ICN Pharmaceuticals Inc., Irvine, Calif.; IgG Sorb was obtained from the Enzyme Center, Boston, Mass.; glusulase was obtained from Du Pont Pharmaceuticals, Wilmington, Del.; and Bacto-Agar was obtained from Difco Laboratories, Detroit, Mich.

Plasmid construction, DNA sequencing, and Southern analysis. The DNA fragments (*PEP4*^{x}) cloned by complementation of a *pep4-3* mutation and by overproduction of PrA (27a) were independently sequenced by our laboratories, using the dideoxy chain termination method of Sanger et al. (29). The 2,147-base-pair (bp) fragment from *Eco*RI to *Xho*I, which was identical in sequence for these two pieces of DNA, was analyzed by using the strategy shown in Fig. 1. DNA fragments were subcloned in M13mp vectors (38) by using standard techniques (19). Priming of single-stranded DNA was done either with commercially available universal primers (Bethesda Research Laboratories and New England BioLabs) or with 18-base oligonucleotides synthesized on an Applied Biosystems model 380 DNA synthesizer.

The plasmids used to disrupt the genomic *PEP4* locus were constructed as follows. A 6.9-kilobase-pair (kb) *PEP4*^{x} fragment was obtained by cutting the original genomic clone with *Bam*HI and was inserted into the *Bam*HI site of pUC4 to produce plasmid pP1. A second plasmid, pP2, was obtained by cloning the 2.3-kb region from *Eco*RI to *Stu*I into the *Eco*RI and *Pvu*II sites of pBR322. In both cases the 1.2-kb *Hin*dIII fragment (containing most of the *PEP4*^{x} coding sequence) was then removed and replaced with a 1.1-kb *Hin*dIII fragment containing the *URA3* gene (1), resulting in plasmids pP3 and pP4, respectively. A similar substitution in *PEP4*^{x} was constructed in pP1 by using the *LEU2* gene. The 2.9-kb *Bgl*II *LEU2* fragment was isolated from YEp13 (1), its ends were blunted with the Klenow fragment, and *Hin*dIII linkers were added. After digestion with *Hin*dIII, this fragment was used to replace the 1.2-kb *Hin*dIII fragment of *PEP4*^{x}, resulting in plasmid pP5. To construct plasmid pTS18, a single-copy autonomously replicating centromere plasmid containing the *PEP4*^{x} gene (CEN-

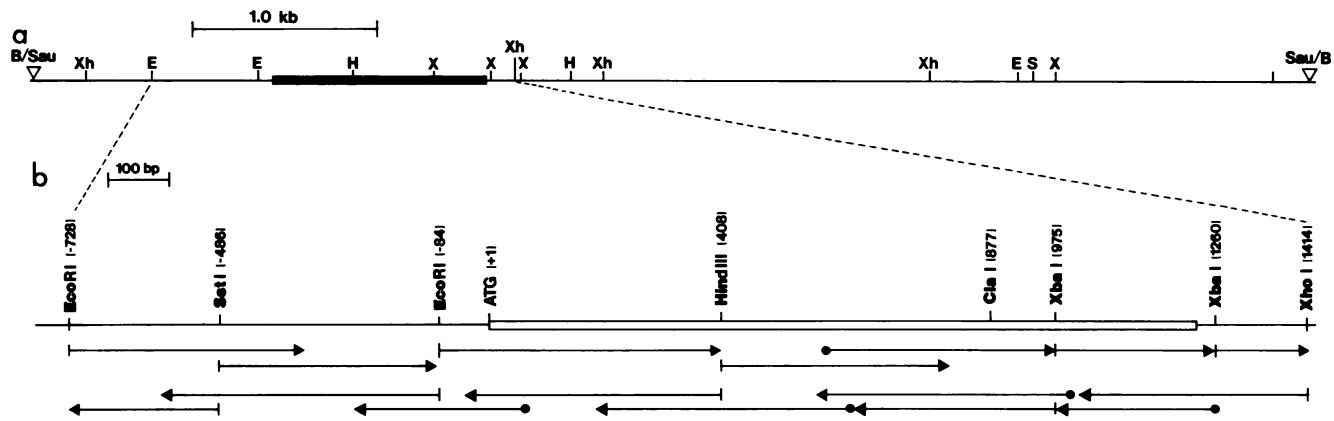

FIG. 1. Restriction map and sequencing strategy for the *PEP4* gene. The sequence corresponding to the coding region is indicated by the solid box on the restriction map of the 6.9-kb *PEP4* cloned fragment (a). The positions of the restriction sites are numbered relative to the adenine residue of the initiating methionine and correspond to the first nucleotide of each restriction site recognition sequence. The sequencing strategy (b) shows the subclones inserted into m13 vectors and sequenced. The arrows indicate the strands sequenced, while the dots indicate that priming was carried out with an 18-base oligonucleotide; 100% of the sequence was determined from both strands. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; Sau, *Sau*3A; S, *Sal*I; X, *Xba*I; Xh, *Xho*I. Symbol: ▽, endpoints of genomic DNA insertion.

PEP4^x), a 4.2-kb *PEP4*^{x} *Bam*HI-*Sal*I (the *Bam*HI site was created during the construction of the genomic library [Fig. 1]) fragment was inserted into YCp50 (1).

Southern analysis was performed as described previously (30), with the following alterations. Yeast genomic DNA was digested to completion with either *Hin*dIII and *Bam*HI or *Xba*I alone, and the fragments were resolved on a 0.8% agarose gel. After transfer to nitrocellulose, the blot was probed with $\sim 2 \times 10^6$ dpm of a nick-translated fragment at 65°C in a 6× SCP solution (0.6 M NaCl plus 150 mM Na_2HPO_4 plus 10 mM EDTA). The blot was washed in 2× SCP at 45°C, dried, and autoradiographed.

Preparation of crude extracts and enzyme assays. Extracts were prepared from log-phase cells by using the buffer conditions and volumes described by Jones et al. (15). Cells were broken by adding 0.1 g of acid-washed glass beads (250 to 300 μm) and 40 μl of 50 mM Tris hydrochloride (pH 7.6) per 10 A_{600} units of cells ($\sim 10^8$ cells) to the cell pellet, followed by vigorous vortexing for 4 min in glass test tubes (13 by 100 mm). The extract was clarified by pelleting the insoluble material for 15 min in a microfuge (Fisher Scientific Co., Pittsburgh, Pa.) at 4°C. CPY (31a), nonspecific alkaline phosphatase (25), PrB (15), and total protein (18) were assayed as described elsewhere. PrA was assayed as described by Wiemken et al. (36) by using denatured hemoglobin prepared as described by Jones et al. (15). Blanks for each PrA assay were prepared by adding perchloric acid to the extract prior to the addition of the hemoglobin substrate.

Protease plate stains. Patches of yeast cells were examined for PrB activity by using a modification of the procedure described by Wolf and Ehmann (37). The modified procedure allowed detection of PrB activity without the requirement for cell lysis mutants. Well-separated patches of yeast cells grown on YEPD agar were overlaid with 10 ml of a solution containing 0.6% agar, 50 mM morpholineethanesulfonic acid (pH 5.0), 0.5% Triton X-100, 20 mg of Azocoll per ml, and 0.1% glusulase. The glusulase was added to the heated overlay mixture after cooling to less than 50°C, and the overlay was poured immediately; 200 μl of 50% β-mercaptoethanol was spread over the solidified overlay. The plates were incubated in plastic bags at 37°C for 1 to 3 days. Patches of yeast cells were examined for CPY by using the CPY plate assay with *N*-acetyl-DL-phenylalanine β-naphthyl ester as the substrate (13).

Antibodies. Affinity-purified CPY antibody was prepared as described previously (31). Antiserum to PrA was prepared by injecting rabbits with PrA obtained from Sigma Chemical Co. as described previously for CPY (31). To remove carbohydrate-reactive antibodies from the PrA antiserum, the serum was adsorbed twice to whole yeast cells by suspending 0.5 g (wet weight) of cells in 20 ml of serum and sedimenting the cells. The treated PrA antiserum was subsequently affinity purified on a column charged with PrA as described previously for CPY (31).

Immunoprecipitation. Cells were grown in low-sulfate medium, labeled with $H_2{}^{35}SO_4$ for 30 min, and removed from the labeling medium by sedimentation in a clinical centrifuge. Extracts were obtained by adding 100 μl of 1% sodium dodecyl sulfate to the labeled cells, heating the preparation in a boiling water bath for 3 min, and vortexing with 0.1 g of 250- to 300-μm glass beads for 90 s. The extracts were then diluted, centrifuged for 10 min in a microfuge, and immunoprecipitated with CPY or PrA affinity-purified antibody as described previously (31a). The immunoprecipitates were washed, solubilized, loaded onto a sodium dodecyl sulfate-polyacrylamide gel, and electrophoresed as previously described (31a). The gels were fixed, permeated with sodium salicylate (17) for fluorography, dried, and exposed to Kodak XAR-5 film at −80°C.

RESULTS

Obtaining a *pep4*-complementing plasmid. To determine the role of the *PEP4* gene product in the activation of yeast vacuolar zymogens, a DNA fragment that complemented a *pep4* mutation was cloned and characterized. A *pep4 leu2* yeast strain (SF657-2D) was transformed with a YEp13 (*LEU2*) yeast genomic bank (26), and Leu$^+$ transformants were selected. About 20,000 Leu$^+$ transformants were screened for CPY activity by using a CPY plate staining procedure (13). Plasmid DNAs were isolated from 16 colonies that tested positive with the CPY plate stain and were used to transform *Escherichia coli* to ampicillin resistance. We obtained one plasmid which, when reintroduced into yeast cells, suppressed the CPY deficiency characteristic of a *pep4* strain. Restriction enzyme mapping indicated that this plasmid contains a 6.9-kb insertion of yeast genomic DNA (Fig. 1). Various subclones of this 6.9-kb insertion were constructed to define better the limits of the gene. *pep4* cells were transformed with these subclones, and the resulting transformants were tested for complementation by the CPY plate assay. The coding region was assigned to the approximately 1,500-bp *Eco*RI-*Xho*I restriction fragment. This 1,500-bp fragment and the adjacent 650-bp *Eco*RI fragment were used for DNA sequence analysis.

Nucleotide and predicted polypeptide sequences. The sequencing strategy used for a 2,147-bp region is shown in Fig. 1, and the complete nucleotide sequence is shown in Fig. 2. The sequence included 728 bp of 5′ flanking sequences and 204 bp of 3′ flanking sequences in addition to 1,215 bp of coding DNA. The open reading frame predicted the synthesis of a 405-amino-acid protein with a molecular weight of 44,431.

The PrA structural gene was recently cloned by using an immunological screening procedure that detects cells containing yeast plasmids that cause overproduction of PrA (27a). This gene was sequenced independently of the putative *PEP4* gene, and these fragments were found to be identical over the entire 2,147 bp. The cloned gene was shown to encode PrA since the N-terminal 12 amino acids of the mature protein (determined by conventional amino acid sequencing) are found within the open reading frame of the gene (27a). In addition, the recently obtained complete amino acid sequence of the mature protein (4) is identical to the sequence predicted from the PrA structural gene DNA sequence. Thus, either the *PEP4* gene encodes PrA or the PrA structural gene is not *PEP4* but was cloned because it can complement a *pep4-3* mutation at a high copy number. Therefore, the cloned PrA structural gene will be referred to as *PEP4*^{x} until its relationship to the *PEP4* locus is determined.

From the DNA sequence of the PrA structural gene we predicted the synthesis of a 405-amino-acid protein if initiation of translation occurs at methionine codon 1 (Fig. 2). If this is the case, then PrA is synthesized with a hydrophobic amino terminus (amino acids 6 through 22) that could serve as a signal sequence to direct PrA to the endoplasmic reticulum (34) and thus facilitate the initial stages of transport to the vacuole. The only other methionine in frame with the mature protein is at codon 38. However, this initiation codon would produce a translation product whose molecular weight is inconsistent with that deduced from gel electro-

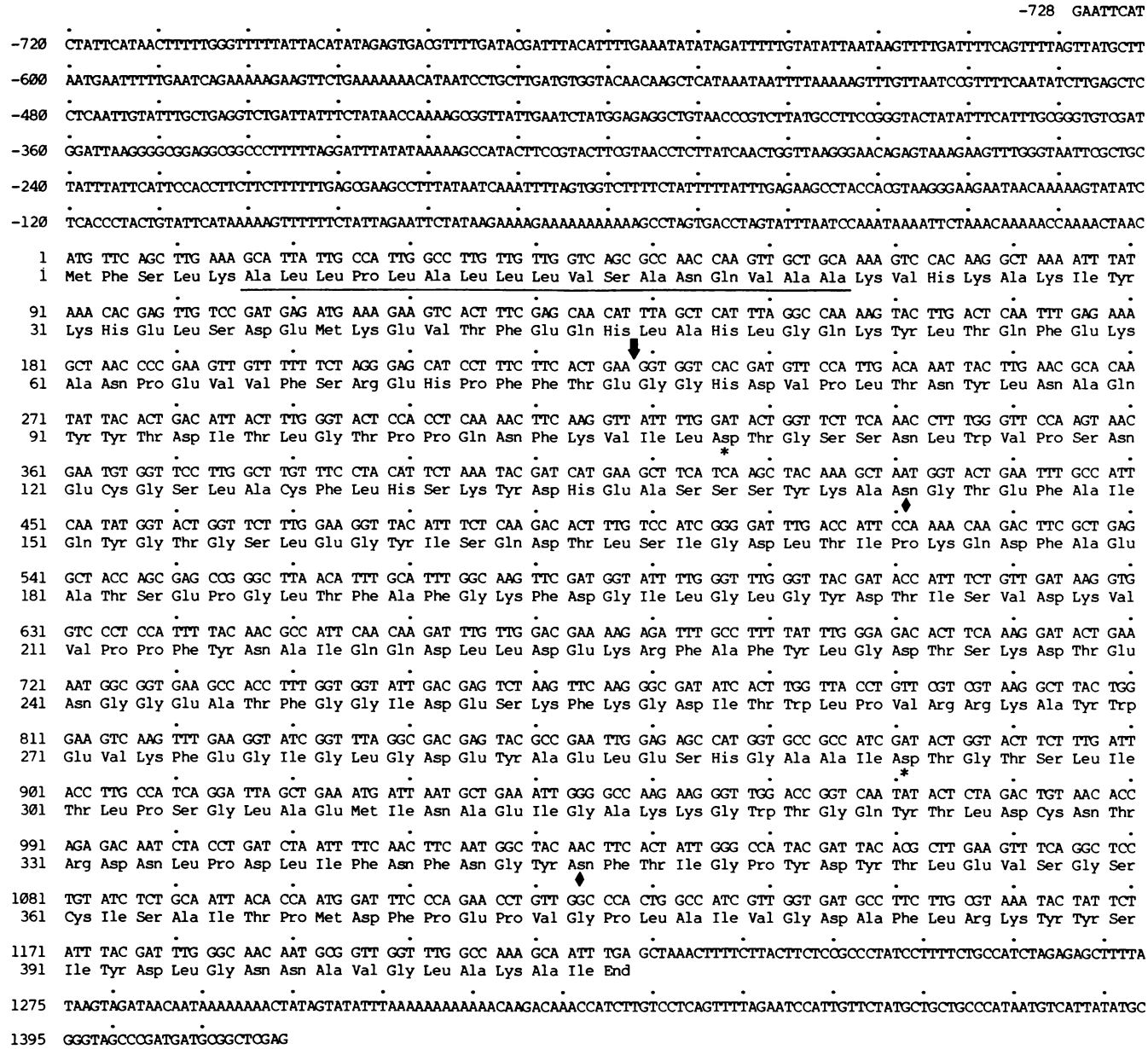

FIG. 2. DNA sequence of *PEP4*. The DNA sequence of the *PEP4* gene from the *Eco*RI site (position −728) to the *Xho*I site (position 1419) and the translated PrA amino acid sequence are shown. The hydrophobic stretch of amino acids near the N terminus is underlined. The arrow indicates the zymogen-processing site. Symbols: ◆, potential glycosylation sites in the protein sequence; *, conserved active-site aspartic acid residues (32).

phoresis studies; furthermore, this translated protein would not have a signal sequence characteristic of proteins that enter the secretory pathway. The amino terminus of the mature protein identified by conventional amino acid sequencing (27a) appears at residue 77 relative to the initiation codon, and this indicates that PrA is synthesized as a precursor (proPrA) which is 76 amino acids larger than the mature enzyme (329 amino acids; molecular weight without carbohydrate, 36,710). A proPrA precursor that is 76 amino acids larger than the mature enzyme is consistent with a predicted molecular weight for proPrA (405 amino acids; molecular weight without carbohydrate, 44,431) that is about 8,000 to 10,000 larger than the molecular weight of mature PrA based on pulse-chase immunoprecipitation studies of PrA in yeast cells (21). The 76-amino-acid propeptide of PrA does not contain any potential sites for asparagine-linked oligosaccharide addition, while two such sites are present in the mature PrA sequence (amino acids 144 and 345). Both of these sites are likely to be glycosylated in vivo since mature PrA contains about 6 kilodaltons of carbohydrate (21, 24), which is consistent with the addition of two asparagine-linked oligosaccharide chains (8).

PrA is homologous to aspartyl proteases. A comparison of the protein sequence of PrA with other polypeptide sequences revealed that PrA is highly homologous to a class of proteins known as aspartyl proteases. All such proteases have an aspartic acid residue at the active site, as exemplified by pepsin and cathepsin D (6, 32). Mature PrA exhibits

```
Y—PrA   1 MFSLKALLPLALLLVSANQVAAKVHKAKIYKHELSDEMKEVTFEQHLAHL 50
H—CD    1 -MQPSS.....C.-L.APAS.L.RIPLHKFTSIRRT.S..--------- 39
H—PG    1 ---M.W..L.G.VAL.ECIMYKVPLIR.KSLRRTLS.RGLLKDFLKKHN. 47

Y—PrA  51 GQKYLTQFEKANPEVVFSREHPFFTEGGHDVPLTNYLNAQYYTDITLGTP 100
H—CD   40 -GGSVEDLIAKG.VSKY.QAV.AV...PIPEV.K..MD....GE.GI... 88
H—PG   48 NPARKYFPQWEA.TL----------VDEQ..E...DME.FGT.GI... 85

Y—PrA 101 PQNFKVILDTGSSNLWVPSNECG--SLACFLHSKYDHEASSSYKANGTEF 148
H—CD   89 ..C.T.VF.........IH.KLLDI..WI.H..NSDK..T.VK...S. 138
H—PG   86 A.D.T.VF.........VY.S--....TN.NRFNP.D..T.QSTSETV 133

Y—PrA 149 AIQYGTGSLEGYISQDTLS----------IGDLTIPKQDFAEATSEPGL 187
H—CD  139 D.H..S...S..L....V.VPCQSASSASAL.GVKVER.V.G...KQ..I 188
H—PG  134 SIT.....MT.ILGY..VQ----------V.GISDTN.I.GLSET...S 172

Y—PrA 188 TFAFGKFDGILGLGYDTISVDKVVPPFYNAIQQDLLDEKRFAFYLGDTSK 237
H—CD  189 ..IAA.......MA.PR...NN.L.V.D.LM..K.V.QNI.S...-SRDP 237
H—PG  173 FLYYAP.......A.PS..SSGAT.V.D.IWN.G.VSQDL.SV..---.A 219

Y—PrA 238 DTENGGEATFGGIDESKFKGDITWLPVRRKAYWEVKFEGI-GLGDEYAEL 286
H—CD  238 .AQP...LML..T.SKYY..SLSY.N.T.....Q.HLDQVEVASGLTLCK 287
H—PG  220 .DQS.SVVI.....S.YYT.SLN.V..TVEG..QITVDS.TMN.EAI.CA 269

Y—PrA 287 ESHGAAIDTGTSLITLPSGLAEMINAEIGAKKGWTGQYTLDCNTRDNLPD 336
H—CD  288 .GCE.IV.......MVG.VDEVRELQKA...VPLIQ.E.MIP.EKVST..A 337
H—PG  270 .GCQ.IV.....L.G.TSPIA..QSD...SENSD.DMVVS.SAISS... 319

Y—PrA 337 LIFNFNGYNFTIGPYDYTLEVS----GSCISAITPMDFPEPVGPLAIVGD 382
H—CD  338 ITLKLG.KGYKLS.E....K..QAGKTL.L.GFMG..I.P.S...W.L.. 387
H—PG  320 IV.TI..VQYPVP.SA.I.QSE----.....GFQG.NL.TES.E.W.L.. 365

Y—PrA 383 AFLRKYYSIYDLGNNAVGLAKAI-- 405
H—CD  388 V.IGR..TVF.RD..R..F.E.ARL 412
H—PG  366 V.I.Q.FTVF.RA..Q....PVA-- 388
```

FIG. 3. Alignment of amino acid sequences of yeast proPrA (Y-PrA), human cathepsin D (H-CD), and human pepsinogen (H-PG). The dots in the lower two sequences indicate identity with the yeast PrA sequence. The dashes indicate insertions of gaps at positions at which there are no homologous residues. The arrow indicates the zymogen-processing site for the proteases. Potential glycosylation sites are indicated by diamonds. Boxes are placed around the following homologies: signal sequence, zymogen-processing site, conserved active-site aspartic acid residues, and the first glycosylation site.

40% amino acid homology (132 of 329 residues) to human pepsin and 46% homology (150 of 329 residues) to human cathepsin D (5) (Fig. 3; Table 2). This degree of homology is significant, as the average level of amino acid identity within the aspartyl protease family is about 40 to 50% (6, 32). For instance, pepsin and cathepsin D show 49% homology at the amino acid level, while pepsin and renin share 39% identical residues (6). It is noteworthy that the amino acids near the active-site aspartic acid residues (Asp-109 and Asp-295) are conserved in PrA.

The propeptide segment of PrA shares more homology with the propeptide of cathepsin D than with the propeptides of secreted aspartyl proteases (Fig. 3 and Table 2). It is interesting to note that the bulk of the propeptide homology between PrA and cathepsin D is in two regions. There is a stretch where seven of eight amino acids are identical (amino acids 7 through 14) in the signal sequences of PrA and cathepsin D, and the zymogen-processing site (Thr · Glu-Gly) is the same in the two proteins. These homologous regions are observed only in proPrA and mammalian lysosomal procathepsin D; they are not found in the propeptides of any of the secreted aspartyl proteases. The conserved processing site may indicate that these two enzymes are activated by a similar mechanism.

TABLE 2. Levels of homology of PrA to aspartyl proteases[a]

Protease compared with proPrA	% Amino acid homology	
	Mature enzymes	Propeptides
Procathepsin D	46	29
Pepsinogen	40	16
Prochymosin	37	21
Prorenin	40	17
Avg for secretory aspartyl proteases	39	18

[a] Sequences were obtained from reference 6.

[b] Levels of homology were calculated by adding the number of identical residues in Fig. 3 (dots) and dividing by the total number of residues compared. For example, for mature PrA and cathepsin D there were 150 identical residues out of 329 amino acids (46% homology).

Southern blot analysis of the substituted PrA structural gene. To examine the phenotype of a strain deleted for the PrA structural gene, two different substitutions were constructed. The 1.2-kb *Hin*dIII fragment in the coding region of the gene (Fig. 4) was replaced with either a 1.1-kb *URA3* fragment or a 2.9-kb *LEU2* fragment (Fig. 4). These substitutions were inserted into the yeast genome by the single-step gene replacement procedure (28), after plasmid pP4 was cut with *Eco*RI and *Xho*I and pP5 was cut with *Bam*HI. A Southern blot analysis was carried out to demonstrate that integration of the substituted alleles occurred at the genomic locus corresponding to the cloned gene. When the 1.5-kb *Eco*RI-*Xho*I fragment was used as a probe, wild-type yeast DNA cut with *Hin*dIII and *Bam*HI displayed the expected strongly hybridized bands at 1.2 and 2.2 kb (Fig. 4A, lane 1),

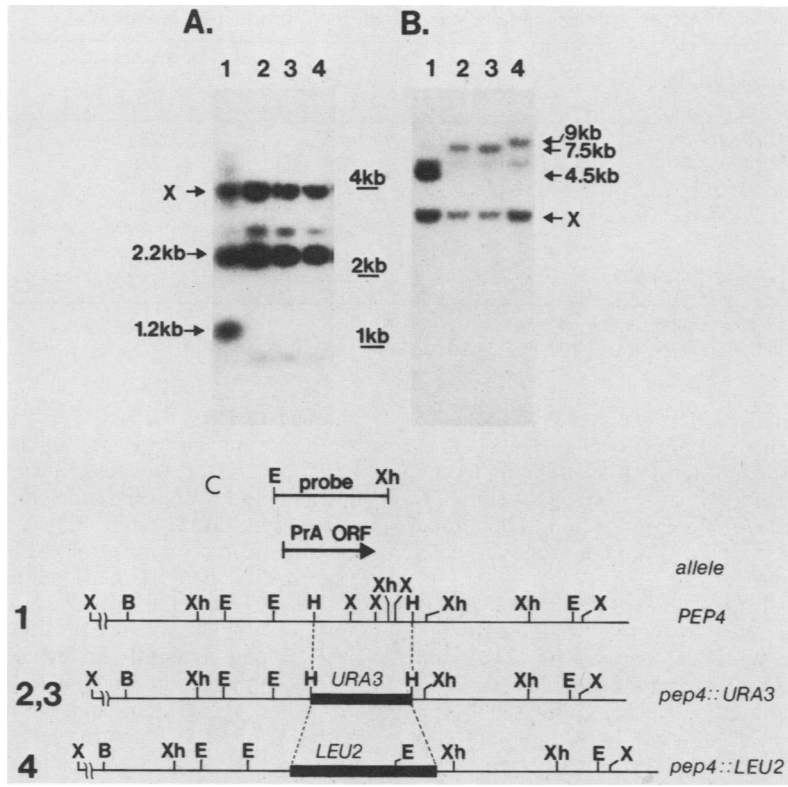

FIG. 4. Southern blot analysis of yeast genomic DNA. Genomic DNA was cut either with *Hin*dIII and *Bam*HI (A) or with *Xba*I (B). Both preparations were probed with a 1.5-kb *Eco*RI-*Xho*I *PEP4* probe (C). The arrows indicate the bands expected from the *PEP4* gene and the strongly hybridizing bands from a non-*PEP4* gene (X). The detailed genomic restriction maps for the *PEP4* (map 1), *pep4*::*URA3* (map 2,3), and *pep4*::*LEU2* (map 4) alleles are shown. The solid boxes are either the *URA3* gene (map 2,3) or the *LEU2* gene (map 4) in the *pep4* substitutions. E, *Eco*RI; Xh, *Xho*I; B, *Bam*HI; X, *Xba*I; H, *Hin*dIII; ORF, open reading frame.

while DNA digested with *Hin*dIII and *Bam*HI from strains with *URA3* or *LEU2* substitutions had only the 2.2-kb band (Fig. 4A, lanes 2 through 4). With the same hybridization probe the expected 4.5-kb band (Fig. 4B, lane 1) and 290-bp band (which cannot be seen in Fig. 4) were observed for wild-type yeast DNA cut with *Xba*I. This same probe hybridized to 7.5- and 9-kb bands from *Xba*I-cut genomic DNA from strains carrying the *URA3* and *LEU2* insertions (Fig. 4B), respectively. Thus, these results demonstrate that both substitutions occurred at the genomic locus corresponding to the cloned gene.

It is clear from these Southern blots that at least two loci hybridized strongly to the nick-translated probes. When the 1.5-kb *Eco*RI-*Xho*I probe was used to hybridize to *Hin*dIII-digested DNA from a wild-type strain, the expected 1.2- and 2.2-kb bands were observed, but in addition at least one other band was observed under high-stringency conditions (Fig. 4A). Multiple bands were also observed when *Xba*I-cut genomic DNA (Fig. 4B) was probed under stringent hybridization conditions. These results suggest that other yeast genes are homologous to the PrA structural gene, possibly indicating the presence of a closely related aspartyl protease.

Complementation analysis of the *PEP4*^{x} and *PEP4* genes. To determine whether the *PEP4* gene indeed codes for PrA, it was necessary to ascertain whether the integrated PrA structural gene substitutions (Δ*pep4*^{x}) constructed in vitro complemented the *pep4-3* mutation. Cells carrying the *pep4-3* mutation lack CPY, PrA, and PrB activities and have greatly reduced levels of a nonspecific alkaline phosphatase (10). Table 3 shows the activity levels for these vacuolar enzymes in a representative set of haploids and diploids. As observed previously (27a), a Δ*pep4*^{x} strain has levels of vacuolar enzyme activities similar to those of a *pep4* strain (Table 3). The residual alkaline phosphatase activity is believed to be due to a distinct nonvacuolar cytoplasmic enzyme (10, 36). A heterozygous *PEP4*/Δ*pep4*^{x} strain has vacuolar enzyme levels similar to those of *PEP4*/*pep4-3* diploid. These strains have wild-type (*PEP4* homozygous) levels of CPY and alkaline phosphatase and somewhat-lower-than-wild-type levels of PrA and PrB activities (Table 3). The *PEP4* gene dosage effect on PrA and PrB enzyme activity levels has been observed previously (15). Interestingly, when a *PEP4*/Δ*pep4*^{x} diploid is sporulated, a phenotypic lag is observed in the Δ*pep4*^{x} spores, analogous to what is observed for *PEP4*/*pep4-3* diploids (40). Diploid strains JHRY89 and JHRY90 (Δ*pep4*^{x}/*pep4-3* and *pep4-3*/*pep4-3*, respectively) had undetectable levels of CPY, PrA, and PrB and greatly reduced levels of nonspecific alkaline phosphatase (Table 3). In addition, these diploids failed to sporulate (Table 3), as expected of diploids homozygous for *pep4* (39).

The Pep$^-$ phenotype of yeast strains carrying the *pep4-3* mutation should be complemented by the cloned *PEP4*^{x} gene on a centromere-containing (single-copy) plasmid, as well as when this gene is carried on a 2-μm circle (multiple-copy) plasmid. Table 2 shows that the PrA structural gene in centromere-containing plasmid pTS18 (CEN-*PEP4*^{x}) completely complemented the *pep4-3* mutation, resulting in wild-type levels of the vacuolar hydrolase enzyme activities. Figure 5 shows that *pep4-3* cells also accumulated proCPY (Fig. 5B, lane 1) and lacked immunoreactive PrA (Fig. 5A,

TABLE 3. Vacuolar enzyme activities and sporulation of diploids[a]

Strain	Relevant genotype	PrA activity	CPY activity	Alkaline phosphatase activity	PrB activity	Sporulation[b]
JHRY85	*PEP4/PEP4*	15.5	245	269	13.3	+
JHRY88	*PEP4/pep4-3*	7.9	223	260	10.0	+
JHRY86	*Δpep4/PEP4*	8.0	250	417	7.4	+
JHRY89	*Δpep4/pep4-3*	0.3	24	49	1.0	−
JHRY90	*pep4-3/pep4-3*	0.4	29	59	1.0	−
SF838-1D(YEp24)	*pep4-3*	0.4	3	36	0.2	NA
SF838-1D(pTS18)	*pep4-3*/CEN-*PEP4*	11.1	169	157	9.0	NA

[a] All activities are given in milliunits per milligram of protein.
[b] Sporulation was scored as follows: +, 10 to 30% sporulation; −, <0.1% sporulation. NA, Not applicable.

lane 1). These phenotypes for CPY (10) and PrA (22, 27a) have been observed previously in *pep4-3* cells. However, in *pep4* cells carrying pTS18, CPY and PrA accumulate as the wild-type 57- and 52-kilodalton mature enzymes (Fig. 5), respectively. Therefore, the single-copy plasmid containing the *PEP4*^{x} gene complements all of the phenotypes of cells carrying the *pep4-3* mutation.

PrA structural gene substitutions map at the *PEP4* locus. A strain carrying a marked *PEP4*^{x} locus was constructed (SF838-5A *PEP4*::*URA3*) by cutting pP4 with *Xho*I and transforming the strain to Ura$^+$, such that the *URA3*-marked gene (Fig. 4C) was integrated adjacent to the wild-type chromosomal copy of the *PEP4*^{x} gene by homologous recombination (27). These *URA3*-marked transformants carried a tandem duplication of the *PEP4*^{x} gene, one copy of which had most of the coding region replaced with the *URA3* gene, and the resulting strain was phenotypically Pep$^+$. This construction was necessary since it has been shown that a diploid formed by mating a haploid strain carrying a *pep4*^{x} deletion with a *pep4-3* haploid strain does not sporulate (Table 4) (27a). Strain SF838-5A (*PEP4*::*URA3 ura3-52*) was crossed to a *pep4-3 ura3-52* strain, diploids were sporulated, and asci were dissected. The Ura phenotype was scored on plates lacking uracil, and the Pep phenotype was scored by using a modification of the PrB plate stain method (27a), in which a segregant lacking PrB activity was scored as *pep4*$^-$ (10, 14). In this tetrad analysis only parental ditype asci were obtained (2 Pep$^-$ Ura$^-$:2 Pep$^+$ Ura$^+$) (Table 4). Since the Southern analysis was not carried out on strain SF838-5A (*PEP4*::URA3), it was necessary to determine whether integration of the *URA3*-marked gene occurred at the same locus as in strain ZA521 (Fig. 4C). A tetrad analysis of diploids resulting from crossing strain SF838-5A (*PEP4*::*URA3*) with strain ZA521 (*pep4*::*URA3*) yielded only parental ditype asci (4 Ura$^+$:0 Ura$^-$) (20 asci were analyzed). Taken together, these results demonstrated that the PrA structural gene (*PEP4*^{x}) locus is tightly linked to the *PEP4* locus.

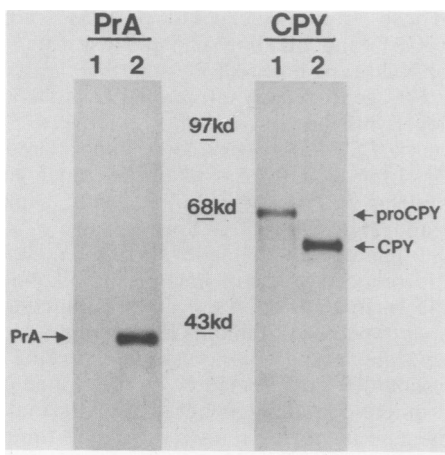

FIG. 5. Single-copy autonomously replicating plasmid containing the *PEP4* gene (pTS18, CEN-*PEP4*) complements the *pep4-3* mutation. CPY and PrA were immunoprecipitated from $H_2{}^{35}SO_4$-radiolabeled crude extracts of *pep4* cells (lanes 1) or *pep4* cells carrying plasmid pTS18 (lanes 2). The arrows indicate the migration positions of PrA, proCPY, and CPY. kd, Kilodalton.

The Southern blot analysis indicated that there is at least one other yeast locus that is highly homologous to the *Eco*RI-*Xho*I PrA structural gene probe (Fig. 4). Since it is possible that homologous integration of the *pep4*::*URA3* DNA could occur in at least two sites, we chose to map a number of independent integrants. Plasmid pP4 (*pep4*::*URA3* DNA in pBR322) (Fig. 4C) was cut at the unique *Xho*I site in *PEP4*, and the linearized plasmid was used to transform strain SF838-5A. Six independent stable Ura$^+$ transformants were crossed to a *pep4* strain, diploids were sporulated, and asci were dissected. A tetrad analysis of all six diploids yielded only parental ditype asci (120 asci were analyzed) (Table 4), indicating that all six independent

TABLE 4. Integrated *PEP4*^{x} gene and *PEP4* exhibit genetic linkage

PEP4 genotype of tester strain (a parent)[a]	Integrant no. (α parent)[a]	No. of parental ditype asci[b]	No. of tetratype and nonparental ditype asci[b]
pep4-3	1	17	0
	2	16	0
	3	19	0
	4	16	0
	5	16	0
	6	11	0
	7	16	0
pep4-Δ1	1	20	0
	3	18	0

[a] Plasmids carrying the PrA structural gene and *URA3* were integrated into the genome of strain SF838-5A (α *PEP4*). Seven independent integrants were analyzed. The Pep phenotype was scored by using the modified PrB plate stain method. Integrants 1 through 4 were crossed with tester strain JHRY20-3CΔ1 (a *pep4-3*), integrants 5 through 7 were crossed with tester strain TSY6-7D (a *pep4-3*), and integrants 1 and 3 were crossed with tester strain ZA521 (a pep4-Δ1).
[b] In crosses with the *pep4-3* tester strains, the parental ditype asci segregated 2 Ura$^+$ Pep$^+$:2 Ura$^-$ Pep$^-$. In crosses with the *pep4-Δ1* tester, the parental ditype asci segregated 4 Ura$^+$:0 Ura$^-$, and tetratype and nonparental ditype asci would segregate 3 Ura$^+$:1 Ura$^-$ and 2 Ura$^+$:2 Ura$^-$, respectively. The Pep phenotype was scored by using the modified PrB plate stain method. Tetrads exhibiting apparent phenotypic lag (43) were grown for about 50 generations and were subsequently scored for the Pep phenotype. Data for only four-spored asci are shown.

homologous integration events had occurred at the same locus, which was tightly linked to *PEP4*.

Taken together, these data conclusively demonstrate that the yeast *PEP4* gene is the structural gene for PrA.

DISCUSSION

It has been hypothesized that the *PEP4* gene of yeasts codes for a vacuolar protease that processes vacuolar zymogens (14) upon their delivery to the vacuole. Cells carrying the *pep4-3* mutation lack PrA, CPY, PrB, aminopeptidase I, vacuolar RNase, and alkaline phosphatase activities. At least two of these vacuolar enzymes (CPY and PrB) are known to accumulate as larger precursors in *pep4* cells (10, 22). Using a combination of cloning, DNA sequencing, complementation analysis, and genetic mapping methods, we found that the *PEP4* gene encodes the well-characterized vacuolar glycoprotein PrA.

It is not known at present whether PrA directly activates vacuolar zymogens by removing their propeptides or whether PrA processes an activating protease(s) that in turn processes other zymogens as part of an activation cascade. In vitro studies (9; unpublished data) have failed thus far to demonstrate processing of proCPY by PrA. It has been demonstrated that PrB is capable of proteolytic conversion of proCPY to an apparent molecular weight very similar to that of mature CPY in vitro (9). In addition, there is in vivo evidence that supports the involvement of PrB as a processing protease (40). Thus, it seems feasible that PrA might activate PrB, which can in turn activate proCPY and perhaps other vacuolar zymogens. On the other hand, none of the PrB mutants that were isolated (*prb1*) seemed to affect CPY activity (9). While this seems to suggest alternate or redundant activation cascades for at least some of the vacuolar enzymes, an analysis of true *prb1* null mutations would more directly address the in vivo role of PrB. Several different mutations (unlinked to *PEP4*, *PRB1*, or *PRC1*) which decrease or abolish both PrB and CPY activities have been described (13). These mutations may identify enzymes that are good candidates for serving in such activation cascades.

Investigators have described *pra1* mutants (16, 23) that are deficient in PrA activity and are reduced in immunoreactive PrA, yet are normal for all other vacuolar enzyme activities. These mutants were identified among cells which were unable to hydrolyze acid-denatured hemoglobin (16, 23). Diploids heterozygous for a *pra1* mutation were shown to have 50% of the wild-type homozygous diploid levels of PrA activity. Based on these results, it was proposed that *PRA1* encodes PrA (14, 16, 23). Since our data demonstrate that *PEP4* is the structural gene for PrA, it is important to understand these earlier genetic results. There are several plausible explanations for the nonpleiotropic phenotypes of the previously identified *pra1* mutations. These mutations might reside within the *PEP4* gene and result in a large reduction in total PrA activity, yet maintain a sufficient level of the active enzyme to allow initiation of the zymogen activation cascade. Such mutations could affect the rate of transcription, translation, stability, activity, or vacuolar delivery of PrA. Consistent with this possibility, it is apparent from the severe phenotypic lag observed for the *pep4-3* mutation (40) that minute amounts of *PEP4* gene product are sufficient to yield near-wild-type levels of vacuolar enzyme activities. Some *pra1* mutations might result in an altered PrA substrate specificity such that PrA could no longer cleave the in vitro substrate (denatured hemoglobin) yet would retain in vivo function. Another possibility is that *pra1* mutations correspond to defects in unlinked regulatory elements necessary for high-level PrA expression. Resolution of this matter must await allelism tests between *pep4* and *pra1* mutations.

Southern analysis of the *PEP4* locus has revealed that at least one non-*PEP4* region of yeast DNA hybridizes strongly to the nick-translated *PEP4* probe. Since the probe used in the Southern analysis was small and consisted primarily of the PrA open reading frame, it is possible that the extra hybridizing bands were due to a protein sharing extensive homology with PrA. These bands do not correspond to the structural genes for either of the proteases CPY (*PRC1* [31a]) or barrier factor (*BAR1* [20; V. L. Mackay, H. Holly, G. Saari, and M. Parker, personal communication]). Therefore, the extra bands in the *PEP4* Southern analysis probably correspond either to an unidentified closely related protease or to a *PEP4* pseudogene.

A comparison of the PrA amino acid sequence with the sequences of other proteins available in the protein sequence data bank revealed that PrA is a member of the aspartyl protease family. The well-characterized members of this family of proteins include pepsin, renin, chymosin, and cathepsin D (6, 32). These proteins are about 40 to 50% identical among themselves, while PrA shares 46 and 40% homology with cathepsin D and pepsin, respectively. All of the eucaryotic aspartyl proteases are either secretory or lysosomal, and all of them except pepsin undergo asparagine-linked glycosylation (6, 32). Interestingly, of the two potential glycosylation sites found in the PrA sequence, the first one, at amino acid 144 (Asn · Gly · Thr), is conserved in human cathepsin D, human renin, and porcine cathepsin D (6). The significance of this conserved glycosylation site is unknown at present.

The levels of homology between the propeptide of PrA and the propeptides of the other aspartyl proteases are less than the levels of homology between the mature proteins. The propeptide of PrA shares only about 18% identity with the propeptides of the secretory aspartyl proteases. However, the PrA propeptide shares about 29% amino acid identity with the propeptide of the human lysosomal aspartyl protease cathepsin D. One of the other major blocks of homology between these two proteases is within the core of the signal sequence, in which seven of eight residues are identical (amino acids 7 through 14). Since five of the seven identical residues are leucine residues, it is possible that the high leucine content of signal sequences (35) makes this homology appear more meaningful than it may actually be. However, the PrA signal sequence is completely unlike the signal sequence of the other sequenced yeast vacuolar enzyme, CPY (30a). Because the human lysosomal cathepsin D signal sequence is cleaved upon translocation (5), it is difficult to visualize the role for a conserved signal sequence, especially since enormous amino acid diversity is tolerated within a functional signal sequence (34).

The proPrA and procathepsin D precursors have identical processing sites (Thr · Glu-Gly), which are not found in secreted proteases, suggesting that these two zymogens may be activated by related processes. In addition, the other homologies in the propeptides might reflect localization determinants common to proPrA and procathepsin D. The fact that cathepsin D is a lysosomal aspartyl protease that exhibits extensive homology to PrA suggests that cathepsin D may play a role analogous to that of PrA in vivo; that is, cathepsin D might be required for proteolytic processing of lysosomal precursor proteins in mammalian lysosomes.

The extensive homology between proPrA and pepsinogen

leads us to suggest an activation mechanism for proPrA similar to that for pepsinogen. Pepsinogen undergoes activation (11) to pepsin by an intramolecular reaction in which the unproteolyzed zymogen cleaves itself (2, 11, 12). Initiation of the autoactivation of pepsinogen requires only that the zymogen be placed in a low-pH environment (pH < 5.0) (2, 11). Since PrA is known to be synthesized as a precursor (21), it is possible that proPrA undergoes autoactivation upon transport to the low-pH environment of the vacuole. Yeast cells that have a mutant copy of the *PEP4* gene accumulate several vacuolar enzymes as inactive precursors (10, 22, 27a). Therefore, since we now know that the *PEP4* gene encodes PrA, it seems entirely plausible that the initiating event in the activation of yeast vacuolar zymogens is the autoactivation of proPrA triggered by the low pH of the vacuole. Experiments to test this hypothesis are under way.

ACKNOWLEDGMENTS

G.A. and G.C.S. thank Marilyn Parker and Caterina Randolph for excellent technical assistance during the cloning experiments and Frank Grant and Patrick O'Hara for advice on sequencing. We thank Vivian MacKay and Thomas Dreyer for making unpublished results available, Beth Blachly for critical reading of the manuscript, and Elizabeth Cooksey for using her expert word-processing skills in the preparation of the manuscript.

J.H.R. and L.A.V. were supported by Public Health Service predoctoral traineeships from the National Institutes of Health. This work was supported by a Public Health Service grant from the National Institute of General Medical Sciences and by a grant from the Chicago Community Trust/Searle Scholars Program to T.H.S.

LITERATURE CITED

1. **Botstein, D., and R. W. Davis.** 1982. Principles and practice of recombinant DNA research in yeast, p. 607–636. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), Molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Bustin, M., and A. Conway-Jacobs.** 1971. Intramolecular activation of porcine pepsinogen. J. Biol. Chem. **246**:615–620.
3. **Creek, K. E., and W. S. Sly.** 1984. The role of the phosphomannosyl receptor in the transport of acid hydrolases to lysosomes, p. 63–82. *In* J. T. Dingle, R. T. Dean, and W. S. Sly (ed.), Lysosomes in biology and pathology. Elsevier/North-Holland Publishing Co., New York.
4. **Dreyer, T., B. Halkjaer, I. Svendsen, and M. Ottesen.** 1986. Primary structure of the aspartic proteinase A from *Saccharomyces cerevisiae*. Carlsberg Res. Commun. **51**:27–41.
5. **Erickson, A. H., G. E. Conner, and G. Blobel.** 1981. Biosynthesis of a lysosomal enzyme. Partial structure of two transient and functionally distinct NH_2-terminal sequences in cathepsin D. J. Biol. Chem. **256**:11224–11231.
6. **Faust, P. L., S. Kornfeld, and J. M. Chirgwin.** 1985. Cloning and sequence analysis of cDNA for human cathepsin D. Proc. Natl. Acad. Sci. USA **82**:4910–4914.
7. **Ferro-Novick, S., W. Hansen, I. Schauer, and R. Schekman.** 1984. Genes required for completion of import of proteins into the endoplasmic reticulum in yeast. J. Cell Biol. **98**:44–53.
8. **Hashimoto, C., R. Cohen, W. Zhang, and C. Ballou.** 1981. Carbohydrate chains on yeast carboxypeptidase Y are phosphorylated. Proc. Natl. Acad. Sci. USA **78**:2244–2248.
9. **Hasilik, A., and W. Tanner.** 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. Conversion of precursor into enzyme. Eur. J. Biochem. **85**:599–608.
10. **Hemmings, B. A., G. S. Zubenko, A. Hasilik, and E. W. Jones.** 1981. Mutants defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **78**:435–439.
11. **Herriott, R. M.** 1939. Kinetics of the formation of pepsin from swine pepsinogen and identification of an intermediate compound. J. Gen. Physiol. **22**:65–78.
12. **James, M. N. G., and A. R. Sielecki.** 1986. Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 Å resolution. Nature (London) **319**:33–38.
13. **Jones, E. W.** 1977. Proteinase mutants of *Saccharomyces cerevisiae*. Genetics **85**:23–33.
14. **Jones, E. W.** 1984. The synthesis and function of proteases in *Saccharomyces*: genetic approaches. Annu. Rev. Genet. **18**:233–270.
15. **Jones, E. W., G. S. Zubenko, and R. P. Parker.** 1982. *PEP4* gene function is required for expression of several vacuolar hydrolases in *Saccharomyces cerevisiae*. Genetics **102**:665–677.
16. **Jones, E. W., G. S. Zubenko, R. R. Parker, B. A. Hemmings, and A. Hasilik.** 1981. Pleiotropic mutations in *S. cerevisiae* which cause deficiency for proteinases and other vacuolar enzymes, p. 182–197. *In* D. Von Wettstein, A. Stenderup, M. Kielland-Brandt, and J. Friis (ed.), Molecular genetics in yeast. Alfred Benzon Symposium, vol. 16. Munksgaard, Copenhagen.
17. **Julius, D., R. Schekman, and J. Thorner.** 1984. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. Cell **36**:309–318.
18. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Manney, T. R.** 1983. Expression of the *BAR1* gene in *Saccharomyces cerevisiae*: induction by the α-mating pheromone of an activity associated with a secreted protein. J. Bacteriol. **155**:291–301.
21. **Mechler, B., M. Müller, H. Müller, F. Muessdoerffer, and D. Wolf.** 1982. *In vivo* biosynthesis of the vacuolar proteinases A and B in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **257**:11203–11206.
22. **Mechler, B., M. Müller, H. Müller, and D. H. Wolf.** 1982. *In vivo* biosynthesis of vacuolar proteinases in proteinase mutants of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **107**:770–778.
23. **Mechler, B., and P. H. Wolf.** 1981. Analysis of proteinase A function in yeast. Eur. J. Biochem. **121**:47–52.
24. **Meussdoerffer, F., P. Tortora, and H. Holzer.** 1980. Purification and properties and proteinase A from yeast. J. Biol. Chem. **255**:12087–12093.
25. **Mitchell, J. K., W. A. Fonzi, J. Wilkerson, and D. J. Opheim.** 1981. A particulate form of alkaline phosphatase in the yeast *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **657**: 482–494.
26. **Nasmyth, K., and S. Reed.** 1980. Isolation of genes by complementation in yeast: molecular cloning of a cell cycle gene. Proc. Natl. Acad. Sci. USA **77**:2119–2123.
27. **Orr-Weaver, T. L., J. W. Szostak, and R. J. Rothstein.** 1981. Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA **78**:6354–6358.

27a. **Rothman, J. H., C. P. Hunter, L. A. Valls, and T. H. Stevens.** 1986. Overproduction-induced mislocalization of a yeast vacuolar protein allows isolation of its structural gene. Proc. Natl. Acad. Sci. USA **83**:3248–3252.

28. **Rothstein, R. K.** 1983. One step gene disruption in yeast. Methods Enzymol. **101**:202–211.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
30. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.

30a. **Stevens, T. H., E. G. Blachly, C. P. Hunter, J. H. Rothman, and L. A. Valls.** 1986. Translocation, sorting and transport of yeast vacuolar glycoproteins. UCLA Symp. Mol. Cell. Biol. **33**:519–536.

31. **Stevens, T. H., B. Esmon, and R. Schekman.** 1982. Early stages

in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell **30**:439–448.

31a. **Stevens, T. H., J. H. Rothman, G. S. Payne, and R. Schekman.** 1986. Gene dosage-dependent secretion of yeast vacuolar carboxy peptidase y. J. Cell Biol. **102**:1551–1557.

32. **Tang, J.** 1979. Evolution in the structure and function of aspartyl proteases. Mol. Cell. Biochem. **26**:93–109.

33. **Trumbly, R., and G. Bradley.** 1983. Isolation and characterization of aminopeptidase mutants of *Saccharomyces cerevisiae*. J. Bacteriol. **156**:36–48.

34. **von Heijne, G.** 1985. Signal sequences: the limits of variation. J. Mol. Biol. **184**:99–105.

35. **Watson, M. E. E.** 1984. Compilation of published signal sequences. Nucleic Acids Res. **12**:5145–5164.

36. **Wiemken, A., M. Schellenberg, and K. Urech.** 1979. Vacuoles: the sole compartment of digestive enzymes in yeast (*Saccharomyces cerevisiae*)? Arch. Microbiol. **123**:23–35.

37. **Wolf, D. H., and C. Ehmann.** 1978. Isolation of yeast mutants lacking proteinase B activity. FEBS Lett. **92**:121–124.

38. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 vectors. Gene **33**:103–119.

39. **Zubenko, G. S., and E. W. Jones.** 1981. Protein degradation, meiosis and sporulation in proteinase-deficient mutants of *Saccharomyces cerevisiae*. Genetics **97**:45–64.

40. **Zubenko, G. S., F. J. Park, and E. W. Jones.** 1982. Genetic properties of mutations at the *PEP4* locus in *Saccharomyces cerevisiae*. Genetics **102**:679–690.

41. **Zubenko, G. S., F. J. Park, and E. W. Jones.** 1983. Mutations in the *PEP4* locus of *Saccharomyces cerevisiae* block the final step in the maturation of two vacuolar hydrolases. Proc. Natl. Acad. Sci. USA **80**:510–514.